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Study of thermal stability of purple acid phosphatase from breadfruit (*Artocarpus communis*) seeds: kinetic and thermodynamic analysis.

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1 SUMMARY

The thermal stability of purple acid phosphatase from breadfruit (*Artocarpus communis*) seeds was investigated by studying the effect of heat treatment over a range of 55°C to 75 °C. Thermal inactivation of this enzyme, evaluated by loss in activity, was apparently followed by first-order kinetics with k-values comprised between 0.011–0.0447 min^{-1}. D and k-values decreased and increased, respectively, with increasing temperature, indicating faster inactivation of purple acid phosphatase at higher temperatures. Ea and Z-values were estimated to 65.405 kJ mol^{-1} and 33.44°C respectively. Thermodynamic parameters were also calculated. All the results suggest that this purple acid phosphatase was relatively resistant to long heat treatments up to 60 °C.

2 INTRODUCTION

Acid phosphatases (Ec 3.1.3.2) produced by both prokaryotic and eukaryotic cells and are presumed to convert organic phosphorus (Ehsanpour and amini, 2003; Amlabu *et al.*, 2009). Phosphate is an important molecule for cellular growth that involved in many different biological reactions (Schachtman *et al.*, 1998). The hydrolysis of phosphomonoesters by phosphatases in biological systems is an important process. This process is linked to energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways (Allan *et al.*, 1994). Purple acid phosphatases represent a distinct category of non-specific acid phosphatases containing a binuclear metal-ion complex at their active site (Vincent *et al.*, 1992; Schenk *et al.*, 1999). They are distinguished from other phosphatases by their characteristic pink or purple colour (due to the presence of a phenolate to metal charge transfer complex), as well as insensitivity to L-tartrate inhibition. In plants, a role in the release of phosphate from organophosphates has been proposed for purple acid phosphatase from *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al.*, 2002). Purple acid phosphatases are widely distributed in plants, and have been found in seeds (Olczak *et al.*, 1997), seedlings (Vasko *et al.*, 2006) and tubers (Shenck *et al.*, 2001; Kusudo *et al.*, 2003). Interest in enzymes also lies in their usages for various biotechnological purposes (Oehmig *et al.* 2007). It was pointed out that some acid phosphatases have been exploited biotechnologically in the food industry (Asano *et al.* 1999) and as tools in environmental bioremediation, removal of heavy metals from metal-polluted soil and

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aqueous wastes (Basnakova *et al.* 1998). Among the industrial enzymes, phytase, a phytate-specific phosphatase was used as a supplement in diets for monogastric animals to improve phosphate utilization from phytate (myo-inositol hexakisphosphate), the major storage form of phosphate in plant seeds (Greiner and Konietzny, 2006). This class of enzymes has also been found increasingly interesting for its use in processing and manufacturing of food for human consumption, particularly because the decline in food phytate results in an enhancement of mineral bioavailability. In a previous study, Konan *et al.* (2008) purified to homogeneity a purple acid phosphatase from breadfruit (*Artocarpus communis*) seeds. This plant belongs to the family Moraceae. It grows in the Tropics, where the fruits are used in a variety of food preparations (Ragone, 2003). The purple acid phophatase showed maximum activity at 55 °C and hydrolyzed broad phosphorylated substrates to various degrees (Konan *et al.*, 2008). In addition, this enzyme showed interesting properties to hydrolyze Sodium phytate. It could cleave phosphate moieties from phytic acid (myo-inositol-hexakisphosphate) present in breadfruit seeds (Konan *et al.*, 2008). So, the physiological function of this enzyme appears to be more important for mammals that feed on breadfruit seeds.Considering these important properties of purple acid phosphatase, the present work aimed to investigate the effect of heat treatment over a range of temperatures from 55 to 75°C on this enzyme. So, determination and analysis of kinetic and thermodynamic parameters were undertaken.

3 MATERIALS AND METHODS

3.1. Enzyme: Purple acid phosphatase used in this study was purified from breadfruit (*Artocarpus communis*) seeds (Konan *et al.*, 2008). This enzyme was homogeneous in on polyacrylamide-gel electrophoresis in the absence of Sodium dodecyl sulphate (SDS).

3.2. Phosphatase assay: Under the standard test conditions, phosphatase activity was measured at 37°C for 10 min in 100 mM acetate buffer (pH 5.5) containing 1.5 mM *para*-Nitrophenylphosphate (*p*NPP). After pre-warming the mixture at 37°C for 5 min, the reaction was initiated by adding 50 µl (6 µg of protein) of enzyme solution. The final volume was 250 µl and the reaction was stopped by adding 2 ml of sodium carbonate (2 %, w/v). Absorbances were measured at 410 nm using a spectrophotometer (SHIMADZU) using *para*-Nitrophenol (*p*NP) as the standard. Under the above experimental conditions, one unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of *p*NP per minute. Specific activity was expressed as units per mg of protein (U/mg of protein).

3.3. Protein determination: Protein was determined according to Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

3.4 Thermal inactivation: The thermal inactivation of purple acid phosphatase was investigated at various constant temperatures from 55 to 75°C after exposure to each temperature for a period of 5 to 60 min. The enzyme was incubated in 100 mM acetate buffer (pH 5.5). Aliquots were drawn at intervals and immediately cooled in ice-cold water. Experiments were performed in triplicate. The residual enzymatic activity, determined in both cases at 37°C under the standard test conditions, was expressed as percentage activity of zero-time control of the untreated enzyme.

3.5 . Kinetic data analysis: Thermal inactivation of purple acid phosphatase can be described by a first-order kinetic model (Terebiznik *et al.*, 1997; Guiavarc'h *et al.*, 2002). The integral effect of an inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Eq. 1:

$$\ln (A_t/A_0) = -kt \quad (1)$$

Where;

A_t is the residual enzyme activity at time t, Ao is the initial enzyme activity; k is the reaction rate constant (min^{-1}) at a given condition. k values were obtained from the regression line of ln (A_t/A_0) versus time as slope. The D-value is defined as a time required, at a constant temperature, to reduce the initial enzyme activity (A_0) by 90 %. For first-order reactions, the

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D-value is directly related to the rate constant k (Eq. 2) (Stumbo, 1973):

D = 2.303/ k (2)

Z (°C) is the temperature increase necessary to induce a 10-fold reduction in D value and follows the Eq 3:

log (D1/D2] = (T2-T1)/Z (3)

where;

T1 and T2 are the lower and higher temperatures in °C or °K; D1 and D2 are D-values at the lower and higher temperatures in min.

The Z values were determined from the linear regression of log (D) and temperature (T).

The temperature of treatment and the rate constant in a denaturation process was related according to the Arrhenius equation (Eq 4 or 5)

$k = Ae^{(-Ea/RT)}$ (4)

or

$\ln k = \ln A - Ea/R \times T$ (5)

where;

k is the reaction rate constant value, A is the Arrhenius constant, Ea is the activation energy (energy required for the inactivation to occur), R is the gas constant (8.31 $Jmol^{-1}K^{-1}$), T is the absolute temperature in °K. When lnk is plotted versus the reciprocal of the absolute temperature, a linear relationship should be observed in the temperature range studied. The slope of the line obtained permitted to calculate the activation energy and the ordinate intercept corresponds to ln A (Dogan *et al.*, 2002). The values of the activation energy (Ea) and Arrhenius constant (A) allowed the determination of different thermodynamic parameters such as variations in enthalpy, entropy and Gibbs free energy, **$\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$,** respectively, according to the following equations (Eq. 6; 7; 8)

$\Delta H^{\#} = Ea - RT$ (6)

$\Delta S^{\#} = R (\ln A - \ln K_B/h_P - \ln T)$ (7)

$\Delta G^{\#} = \Delta H^{\#} - T \Delta S^{\#}$ (8)

Where;

K_B is the Boltzmann constant (1.38 x 10^{-23} J/K),

h_P is the Planck constant (6.626 x 10^{-34} J.s) and T is the absolute temperature.

4 RESULTS AND DISCUSSION

The optimum temperature of purple acid phosphatase purified from breadfruit (Artocarpus communis) seeds was 55 °C (Konan *et al.*, 2008). In this study, the effect of heat treatment over a range of temperatures from 55 to 75°C on purple acid phosphatase was evaluated by determining the residual enzymatic activity. The thermal stability profile of purple acid phosphatase presented in the form of the residual percentage activity is shown in Table 1.

Table 1: Effect of treatment temperature and time on the inactivation of purple acid phosphatase from breadfruit seeds.

Treatment time (min)	Relative activity (%) at each temperature (°C) of heat treatment				
	55	60	65	70	75
5	94.56	88.61	85.15	78.25	68.25
10	90.98	79.09	76.85	69.24	58.72
15	85.28	76.23	68.95	58.96	49.16
20	82.75	70.19	59.93	53.94	38.55
25	81.21	65.27	54.55	45.56	32.81
30	74.452	57.84	50.18	37.55	27.15
35	71.09	53.69	45.38	30.82	22.76
40	65.29	50.65	40.32	26.24	16.38
45	59.65	46.84	37.14	23.63	13.85
50	57.45	42.59	33.05	18.46	10.45
55	53.68	36.08	27.38	14.95	08.25
60	49.05	34.18	25.66	13.04	06.75

The activity of purple acid phosphatase was decreased with increasing heating time (5–60 min) and temperature (55–75°C). Indeed, at temperatures between 55-75°C, heat-denaturation of the enzyme occurred after 5 min of incubation (94.56 to 68.25 %). Although heating at 60°C for 40 min resulted in

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partial (50.65%) inactivation, heating at 75°C for the same period strongly inactivated the enzyme (16.38%). On one hand, the decrease of percentage residual activity at temperatures higher than 55 °C could be explained by the unfolding of tertiary structure of this enzyme to form a secondary structure and on other hand, it could be explained by the chemical modification (Tabatabai, 1982). The linear regressions between the purple acid phosphatase activity and heat treatment time at the different temperatures indicate that the inactivation of the enzyme followed a first-order model (Fig 1). This result was consistent with those reported for acid phosphatase in cod (Johnsen *et al* 2007) and alkaline phosphatase in equine milk (Marchand *et al.*, 2009).

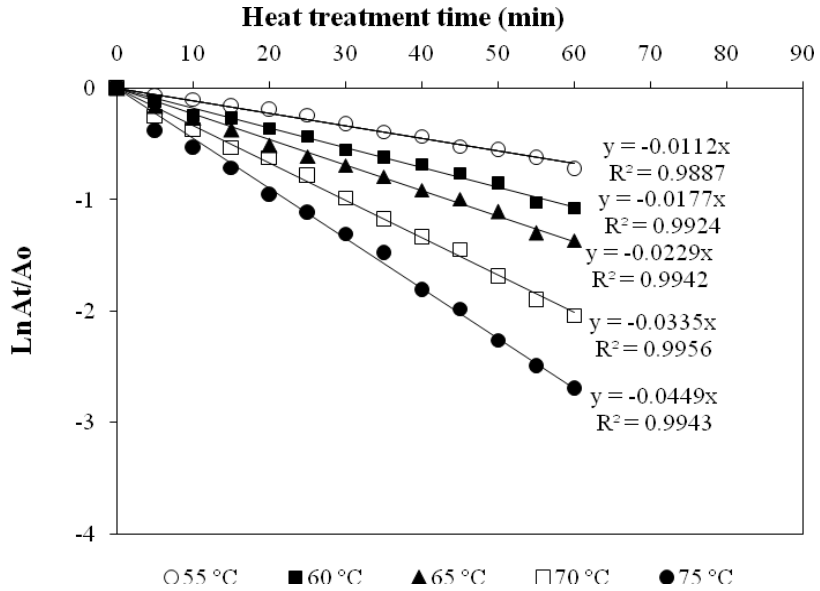

Fig. 1: Thermal inactivation of of purple acid phosphatase from breadfruit seeds in sodium acetate buffer pH 5.5 in the temperature ranged from 55 to 75°C. A0 is the initial enzymatic activity and At the activity at each holding time.

Inactivation rate constants were used to drawn the Arrhenius plot, from which slope activation energy was calculated and found to be 65.405 kJ/mol. This value of activation energy was much higher than that reported for moth bean acid phosphatase (9.44 kJ/mol (Mohamed and Al-Omair, 2010), purple acid Phosphatases from Sweet potato (49.3 kJ/mol (Kusudo *et al.*, 2003), but lower than that for whole acid phosphatase from buckwheat seeds (66.1 kJ/mol (Greiner and Jany, 2002). the higher value found for the activation energy that a higher amount of energy is needed to initiate denaturation (Koffi *et al.*, 2010). As shown in table 2, D-values decreased by increased at temperature. D-values that obtained at 75 and 70°C were about 4 and 3 lower than in comparison to D-value at 55°C, respectively. This remarkable decrease at D-value between 70 and 75°C indicated a potential thermal denaturation of purple acid phosphatase from breadfruit seeds. The effect of temperature on D-values is shown in Fig. 2 and from this representation, the Z-value was calculated and found to be 33.44 °C at 55–75°C (table 2). This Z-value was much higher than that reported for acid phosphatases in cod (6.37°C) (Johnsen *et al.,* 2007) and in ground beef (7.45°C) (Orta-Ramirez *et al.*, 1997). In general, high Z-values mean more sensitivity to the duration of heat treatment and lower Z-values mean more sensitivity to increase in temperature (Barrett *et al.*, 1999). Therefore, the Z-value 33.44°C for breadfruit seeds purple acid phosphatase indicated that this enzyme is more to sensitive to the extension of treatment time than to increase of temperature.

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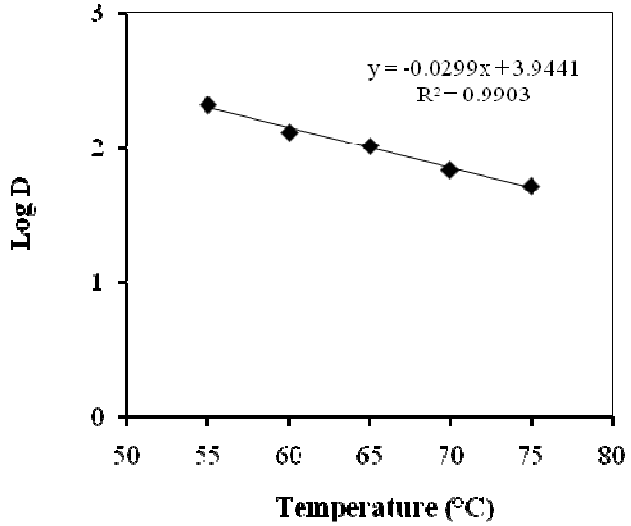

Fig. 2: Effect of temperature on D-values for inactivation of purple acid phosphatase from breadfruit seeds.

Table 2: k, D, Z and Ea-values for thermal inactivation of purple acid phosphatase from breadfruit seeds at temperature range (55–75°C).

Temperature (°C)	Kinetic parameters				
	K (min^{-1})		D (min)	Z (°C)	Ea (kJ mol^{-1})
	Values	R^2			
55	0.011	0.98	205.62		
60	0.0177	0.992	130.11		
65	0.0224	0.974	102.81	33.44	65.405
70	0.0336	0.97	68.54		
75	0.0447	0.97	51.52		

Thermodynamic values of variation in activation enthalpy (ΔH), variation in activation entropy (ΔS), and variation in Gibbs free energy (ΔG) calculated for the different temperatures are shown in Table 3. At temperatures of 55–75°C, the average values of ΔH, ΔS and ΔG were respectively 62.596 (kJ mol^{-1}), -83.560 (J mol^{-1} K^{-1}) and 90.840 (kJ mol^{-1}). The ΔH value was much higher than that reported for acid phosphatase in soil immobilized on clay minerals (13.52 kJ/mol (Rao *et al.,* 2000). The value of ΔH is related to the events necessary to the formation of transition state (Cornish-Bowdel, 1979). Moreover, positive values of ΔH indicate that denaturation of purple acid phosphatase is an endothermic reaction. The ΔS value of purple acid phosphatase was smaller than that reported for *Eschericha coli* alkalin phosphatase (-65.85 J mol^{-1} K^{-1} (Zhang *et al.,* 2003). The negative values observed for the variation in entropy indicate that there are no significant processes of aggregation, since, if this would happen, the values of entropy would be positive (Anema and McKenna, 1996).

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Table 3: Thermodynamics parameters of purple acid phosphatase from breadfruit seeds under heat treatment between 55 to 75°C (assuming a 1st-order kinetic model).

Temperature(°C)	Thermodynamic parameters		
	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
55	62.679	-83.311	90.005
60	62.638	-83.438	90.423
65	62.596	-83.562	90.840
70	62.555	-83.684	91.259
75	62.513	-83.804	91.677
Mean	62.596	-83.560	90.840

Concerning the free energy (ΔG), which is a measure of the spontaneity of the inactivation processes. This value was positive at all temperatures for purple phosphatase and this indicates that the inactivation processes was not spontaneous. Based on this study, it is concluded that thermal inactivation of purple acid phosphatase could be described by a firs-order kinetic model. D-, Z-, k-values, and the high values obtained for activation energy and change in enthalpy indicated that a high amount of energy was needed to initiate denaturation of purple acid phosphatase, most likely due to its stable molecular conformation. This high thermostability may be taken into account when thermal treatments are used to obtain processed products derived from breadfruit (Artocarpus communis) seeds.

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